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Matrix metalloproteinases (MMPs) are thought to play important regulatory roles in normal development and disease. In addition to their established role in tumor invasion and metastasis, recent studies using mice that overexpress or lack specific MMPs or their inhibitors indicate that several MMPs can influence early tumor development. Our data indicate that stromal MMPs play an important role in tumor establishment. The coinjection of mouse embryo fibroblasts (MEFs) fosters human breast carcinoma cell growth in nude mice, whereas isogenic MEFs lacking either MMP-2 (gelatinase-A) or MMP-3 (stromelysin-1), but not MMP-9 (gelatinase-B), are deficient in their ability to foster this growth. This occurs even though the tumor cells and the murine hosts retain the ability to express these genes. This supports the emerging thesis that stromal signals and enzymes are an important factor in early tumor development. In addition, MMP-2 and MMP-3 are upregulated and activated during normal pubertal branching morphogenesis of the murine mammary gland. Transgenic mice that overexpress MMP-3 in mammary gland show increased ductal branching and mice null for MMP-3 show significantly diminished branching, whereas mice lacking MMP-2 have retarded ductal elongation. Thus MMP-2 and MMP-3 play critical roles in both tumor development and normal mammary gland morphogenesis.

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B. Wiseman

B. WISEMAN 06.27.00.

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Introduction

Breast tumors mostly arise in epithelial cells as carcinomas, however there is a growing body of evidence that reciprocal signals between carcinoma cells and adjacent stromal cells are likely to influence tumor evolution. Human conditions such as ulcerative colitis in which inflammation causes the development of a highly reactive stroma or juvenile polyposis syndrome, in which expansion of the stromal compartment results in hamartomatous polyps of the colon, can result in carcinomas. Experimentally similar phenomena have been demonstrated. Stromal fibroblasts are able to promote non-tumorigenic but immortalized epithelial cell-lines or barely tumorigenic carcinoma derived cell-lines to form significant tumors when recombinants are grown subcutaneously or as a graft under the kidney capsule of immunocompromised mice. By using these methods and also utilizing *in vitro* systems we hope to demonstrate that the stroma does have a role in breast tumor promotion and to dissect what factor/s it may contribute. Matrix metalloproteinases (MMPs), stromally expressed metzincins, are thought to play important regulatory roles in normal development and disease through their ability to alter the cellular microenvironment. Accumulating evidence from transgenic and knockout mice suggests that these stromal enzymes influence early carcinogenesis in addition to their established role in invasion and metastasis. Mice null or overexpressing specific MMPs are respectively more resistant or sensitive to tumor promoting agents. Furthermore, overexpression of MMP-3 in the mammary glands of transgenic mice predisposes these mice to mammary carcinomas in the absence of tumor promoters. In addition, MMPs are thought to have a role in normal mammary development. MMP-2 and MMP-3 are upregulated and activated during normal pubertal branching morphogenesis of the murine mammary gland. We have demonstrated that these proteases have distinct roles in ductal development of the mammary gland.

Body

Stromal Factors Can Promote to Tumor Formation

One goal for this research proposal was to develop *in vivo* and *in vitro* systems to study the promotion of mammary epithelial tumorigenesis by stromal factors. Our initial approach has been to use a mammary carcinoma cell-line, MCF7 that is reportedly poorly tumorigenic *in vivo*, but whose tumorigenicity increases if grown in the presence of fibroblasts from a variety of origins. We have shown that murine embryo fibroblasts (MEFs) are able to promote the tumorigenic potential of MCF7 cells when injected subcutaneously into immunocompromised nude mice. After 6 weeks, MCF7 cells formed average tumors of 53 mg if grown alone in the presence of matrigel, in contrast to MCF7 cells combined with MEFs in matrigel which formed tumors with an average mass of 110 mg ($p<0.03$). This system should provide a good model with which to investigate the stromal influence on tumor formation. Initial data indicates a very significant increase in MCF7 cell proliferation (through assessment of the cell proliferation marker PCNA) in very early tumors (6 days) with the presence of MEFs compared to those without ($p<0.0001$). In addition, 6 day old MCF7 cell recombinants in the presence of MEFs show faint signs of angiogenesis through immunohistochemical analysis of the endothelial cell marker, PECAM or CD31, whereas this marker is not evident in implants containing only MCF7 cells. Work is continuing to elucidate differences between these tumors that may provide clues to the mechanisms promoting tumor growth, including assessing different endothelial cell markers, markers of differentiation, cell death rates and *in situ* zymography at a variety of time points.

A Requirement for MMPs in the Stromal Promotion of Tumorigenesis

As mentioned above, candidate effectors of tumor promotion may be the matrix metalloproteinases (MMPs). Hence we utilized our laboratory's transgenic mice which are

either null for specific MMPs or overexpress tissue inhibitor of MMPs-1 (TIMP-1) to generate MEFs in order to find out whether MMPs indeed have a role in the stromal promotion of tumorigenesis We are currently in the process of subcutaneously implanting knockout MEFs (or isogenic wild type(wt) MEFs) with MCF7 cells to discover whether the presence of particular MMPs is required for tumor promotion. Our initial results look promising. MEFs lacking MMP-3 or MMP-2 were significantly retarded in their abilities to promote tumor take as compared to isogenic wtMEFs (with p values of 0.02 and 0.01 respectively). In each case the tumors that did form were not significantly different in size than those that grew with wtMEFs. Thus it appears that stromally expressed MMPs may be important in the establishment of tumors. Interestingly, we see this effect even though the murine host and the MCF7 cells both are able to express the MMP that is lacking in the MEFs. In addition, host stromal cells enter into the tumor plug (as evidenced by immunohistochemical analysis for the mesenchymal marker, vimentin), but this does not appear to be sufficient for tumor establishment. This effect on tumor establishment may be due to a lack of early filtration of endogenous fibroblasts, thus far we have only looked at 6 days. Alternatively, mouse embryo fibroblasts may have different properties from those found in adult mouse skin or may exclude endogenous fibroblasts. We are currently attempting to distinguish between host and transplanted fibroblasts by their different HLA complexes within the tumors to see whether host fibroblasts are specifically excluded from the MEF/MCF7 tumors. We also plan to use fibroblasts of different origins such as skin and mammary gland to determine whether fibroblast origin is an important factor in tumor promotion. These experiments are in their early stages and need to be repeated with different batches of MEFs. In addition, to test whether the effect of MEFs and the requirement for MMPs therein, is a common phenomenon in mammary cell tumor development, the response of other mammary cell-lines to MEFs will be tested. Currently we plan to test the very poorly tumorigenic MCF10, BT-10 and T47D human cell-lines, MBA-MD-231 cells which are highly tumorigenic and EPH4 cells which are a murine mammary cell-line which has been shown to

increase in tumorigenicity in the presence of exogenous MMP-3. Moreover, to establish that the MMP deficiency of the MEFs is responsible for the difference in tumor promotion we will attempt to rescue the tumor promoting effects of the wtMEFs by re-expressing the deficient MMP in the null MEFs.

Establishment of a Co-culture System

To allow us to investigate the mechanisms of stromal promotion of tumorigenesis in more detail we wish to re-capitulate the effects we see *in vivo* in the context of an *in vitro* co-cultivation system. For some studies we have used a direct co-culture system in which fibroblasts are labeled with a fluorescent dye to distinguish them from the epithelial cells both visually and through fluorescent activated cell sorting (FACS). We are currently looking for a marker of transformation that correlates with the ability of MEFs to promote tumorigenesis. Possibilities are proliferative capacity; anchorage independent growth; the ability to migrate and the ability to invade through a basement membrane. DNA synthesis assays are currently being optimized to allow differentiation between epithelial cells and fibroblasts undergoing S-phase using the fluorescent dye and fluorescently labeling incorporated BrdU. We have not observed any increase in the anchorage independent ability of MCF7 cells to grow in soft agar in the presence of MEFs. However fibroblasts signals may be transmitted through breakdown or release of matrix bound proteins in the basement membrane and we would see an effect using matrigel or collagen as a substrate. Those experiments are currently being performed, as are invasion and migration assays. In addition, we have shown *in vivo* that proteases are an important factor in the ability of MEFs to promote tumorigenesis. Co-incubation of epithelial cells with fibroblasts alters protease expression and activation in comparison with these cells grown alone. We have seen interesting differences in protease expression through gelatin and casein zymography when MEFs are directly co-cultured with MCF7 or T47D cells in different proportions. Thus the cells are able to signal and respond to signals from the other cell type. We are still working to identify the specific proteases involved through the use of inhibitors specific to certain classes of

proteases, western blot and by using fibroblasts null for specific MMPs. Thus both cell types are able to signal and respond to signals from the other cell type.

Distinct Roles for MMP-2 and MMP-3 in Mammary Gland Morphogenesis

In order to understand the role of MMPs in breast cancer, it is also important to understand their role in normal breast development. To this end, mammary development in mice deficient in MMPs has been examined in collaboration with others in the laboratory. We have shown that overexpression of Timp-1 in transgenic mice or treatment of mice with synthetic MMP inhibitors retards ductal elongation and branching in the pubescent mammary gland. In addition, through examination of mice null for specific MMPs, the specific requirement of a single MMP for each of these properties has been elucidated. MMP-3 has no effect on ductal penetration but its overexpression increases and its absence diminishes branching of the ducts. Conversely MMP-2 is required for ductal penetration. MMP-2 null mice up to 50 days old have shorter ducts than heterozygotes, but normal branching patterns. However by 10 weeks of age MMP-2 deficient glands have filled the fat pad indicating that these mice can overcome the lack of MMP-2 by an alternative, as yet unknown mechanism. Interestingly, MMP-9 null mice have normal ductal development indicating that the requirements for MMP-2 and MMP-3 are specific. Thus we have separated two distinct processes in mammary gland ductal development and described a requirement for distinct proteases in these processes.

Key Research Accomplishments.

- Development of *in vivo* system for studying stromal promotion of tumor growth
- Demonstrated fostering of tumor take and growth by mouse embryo fibroblasts (MEFs) with subcutaneously injected MCF-7 cell recombinants.
- Observed a significant increase in cell proliferation in early MCF-7 recombinants in the presence of MEFs over recombinants with MCF-7cells alone.
- Observed more early angiogenesis in MCF-7 recombinants in the presence of MEFs over recombinants with MCF-7cells alone.
- Identified a requirement for MMP-3 and MMP-2, but not MMP-9 in fibroblasts, and thus the promotion of tumor take by the stroma.
- Development of *in vitro* co-cultivation systems
- Identified differences in secreted gelatinolytic and caseinolytic activities when breast carcinoma epithelial cells are co-cultured with fibroblasts
- Identified requirement for gelatinase A in ductal invasion of the pubescent murine mammary gland

Reportable Outcomes

Published Abstracts (For platform presentations the presenting author is underlined)

B.S. Wiseman, M.D. Sternlicht, M. Sciabica & Z.Werb. Roles for Matrix Metalloproteinases in Mammary Tumorigenesis and Morphogenesis. VIIIth International Congress of the Metastasis Research Society. Abstract accepted. September 24-27 2000.

B.S. Wiseman, M.D. Sternlicht, M. Sciabica & Z.Werb Roles for Matrix Metalloproteinases in Mammary Morphogenesis and Tumorigenesis.. Hannah Symposium 2000:The Natural History of Breast Cancer. Abstract accepted. September 6-8 2000.

M.D.Sternlicht, **B.S. Wiseman**, M.Simian, M.J.Bissell, Z.Werb. Stromal Metalloproteinases in Morphogenesis, Remodelling and Cancer. Proc. ENDO 2000 The Endocrine Society 82nd Annual Meeting, 17 (# 57) June 23, 2000.

J.L. Rinkenberger, M.D. Sternlicht, **B.S.Wiseman**, M.Sciabica, K. Holmbeck, J. Wiesen, Z. Werb. Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in the Stroma Regulate Mammary Ductal Morphogenesis.. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract # Z-10. June 8, 2000.

Training

- UCSF Committee on Animal Research/Animal Care Facility Training and Certification Program
- Animal Care Facility Training Workshop on animal handling and aseptic techniques
- Laboratory for Cytometric Analysis Flow Cytometry Course

Roles for Stromal Matrix Metalloproteinases in Mammary Tumorigenesis and Morphogenesis

B.S. Wiseman, M.D. Sternlicht, M. Sciabica & Z. Werb

Matrix metalloproteinases (MMPs) are thought to play important regulatory roles in normal development and disease. In addition to their established role in tumor invasion and metastasis, recent studies using mice that overexpress or lack specific MMPs or their inhibitors indicate that several MMPs can influence early tumor development. Although most tumors are epithelial in origin, the majority of MMPs are expressed by the stroma and alter the cellular microenvironment. Our data indicate that stromal MMPs play an important role in tumor establishment. The coinjection of mouse embryo fibroblasts (MEFs) fosters human breast carcinoma cell growth in nude mice, whereas isogenic MEFs lacking either MMP-2 (gelatinase-A) or MMP-3 (stromelysin-1), but not MMP-9 (gelatinase-B), are deficient in their ability to foster this growth. This occurs even though the tumor cells and the murine hosts retain the ability to express these genes. This supports the emerging thesis that stromal signals and enzymes are an important factor in early tumor development. In addition, MMP-2 and MMP-3 are upregulated and activated during normal pubertal branching morphogenesis of the murine mammary gland. Transgenic mice that overexpress MMP-3 in mammary gland show increased ductal branching and mice null for MMP-3 show significantly diminished branching, whereas mice lacking MMP-2 have retarded ductal elongation. Thus MMP-2 and MMP-3 play critical roles in both tumor development and normal mammary gland morphogenesis.

Roles for Matrix Metalloproteinases in Mammary Morphogenesis and Tumorigenesis

B.S. Wiseman, M.D. Sternlicht, M. Sciabica & Z. Werb

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Matrix metalloproteinases (MMPs) are thought to play important regulatory roles in normal development and disease. MMP-2 (gelatinase-A) and MMP-3 (stromelysin-1) are upregulated and activated during normal pubertal branching morphogenesis of the murine mammary gland. Transgenic mice that overexpress MMP-3 in mammary gland show increased ductal branching and mice null for MMP-3 show significantly diminished branching, whereas mice lacking MMP-2 have retarded ductal elongation. MMP-9 (gelatinase-B) is not upregulated during mammary development, nor does its absence alter ductal development. Thus proper development of the mammary gland requires co-ordinated action of at least two, and possibly more, members of the MMP family. In addition to their important roles in development, MMPs also have an established role in tumor invasion and metastasis. Moreover, recent studies using mice that overexpress or lack specific MMPs or their inhibitors indicate that several MMPs can also influence early tumor development. Most tumors are epithelial in origin and yet the majority of MMPs are expressed by the stroma and alter the cellular microenvironment. Thus reciprocal signals between carcinoma cells and adjacent stromal cells are likely to influence tumor evolution. Our data indicate that stromal MMPs play an important role in tumor establishment. The coinjection of mouse embryo fibroblasts (MEFs) fosters human breast carcinoma cell growth in nude mice, whereas isogenic MEFs lacking either MMP-2 or MMP-3, but not MMP-9, are deficient in their ability to foster this growth. This occurs even though the tumor cells and the murine hosts retain the ability to express these genes. This supports the emerging thesis that stromal signals and enzymes are an important factor in early tumor development. Thus MMP-2 and MMP-3 play critical roles in both tumor development and normal mammary gland morphogenesis.

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Keywords: Mammary Development, Matrix Metalloproteinases, Mammary Cancer

STROMAL METALLOPROTEINASES IN MORPHOGENESIS, REMODELING AND CANCER.

Mark D. Sternlicht [1], Bryony S. Wiseman [1], Marina Simian [2], Mina J. Bissell [2] and Zena Werb [1]. [1]Dept. of Anatomy, University of California-San Francisco, San Francisco, CA; and [2]Life Sciences Div., Lawrence Berkeley National Laboratory, Berkeley, CA.

At least 19 matrix metalloproteinases (MMPs) influence various aspects of mammalian development, health and disease. In the mammary gland, MMP2/gelatinase A (GelA) and MMP3/stromelysin-1 (Str1) are upregulated and activated during pubertal branching morphogenesis and postlactational involution. MMPs are required for branching of cultured mammary organoids and recombinant Str1 suffices to promote branching in the absence of growth factors. Analysis of transgenic and knockout mice reveals that Str1 indeed regulates mammary branching in vivo, whereas GelA regulates ductal penetration and elongation within the mammary fat pad. MMP9/gelatinase B is not normally upregulated at puberty and, as expected, its absence in null animals has no effect on mammary development. Transgenic data also suggest that Str1 plays a key role in the apoptosis and remodeling seen during involution. Stromal MMPs are also consistently upregulated in malignant disease. Indeed, many MMPs were cloned as cancer-specific genes and are key agonists in angiogenesis, invasion and metastasis. In addition, recent studies in MMP-overexpressing and -deficient mice indicate that several MMPs can influence early cancer development, but most such studies have required carcinogens or pre-existing mutations to initiate tumorigenesis. We, however, have observed spontaneous premalignant and malignant lesions in the mammary glands of mice with a mammary-targeted Str1 transgene. These changes were absent in nontransgenic littermates and were quenched by the co-expression of a tissue inhibitor of metalloproteinases-1 transgene. Moreover, mouse embryonic fibroblasts foster human breast cancer cell growth in vivo, yet Str1-null fibroblasts do not; and an autoactivating Str1 can convert functionally normal mammary epithelial cells into invasive and tumorigenic mesenchymal-like cells, and can modulate the expression of genes that control cancer development. Thus by altering the cellular microenvironment, Str1 can act as a natural tumor promoter and can enhance cancer susceptibility.

Disclosure: There is no disclosure information to present

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MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES IN THE STROMA REGULATE MAMMARY DUCTAL MORPHOGENESIS.

J. L. Rinkenberger^, M. D. Sternlicht^, B. S. Wiseman^, M. Sciabica^, K. Holmbeck*, J. Wiesen^, H. Birkedal-Hansen*, Z. Werb^.

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Mammary gland ductal morphogenesis begins in late fetal development, continues slowly during the early post-natal period and accelerates in response to ovarian hormones at the onset of puberty. It is well known that ductal epithelial cell morphogenesis is regulated by stromal factors in the surrounding fat pad. Here we show that stromal matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), influence ductal elongation and branching by regulating extracellular matrix remodeling. Transgenic mice that constitutively over-express TIMP-1 show diminished ductal elongation and branching, and branching of cultured mammary organoids is blocked by synthetic MMP inhibitors in vitro. However, these models do not indicate which MMPs are responsible for ductal elongation and branching. MMP-3/stromelysin-1 and MMP-2/gelatinase A are both upregulated at the mRNA and protein levels in the stroma surrounding the developing ducts making them particularly good candidates. In order to determine which MMPs are involved in ductal elongation and branching we have examined mammary gland development in specific MMP over-expressing and null mouse models. MMP-3 has no effect on ductal penetration but its over-expression increases, and its absence significantly decreases, both dichotomous and lateral branching. On the other hand, the MMP-2 null mice exhibit reduced ductal penetration of the fat pad without changes in branching. MMP-9/gelatinase B is not normally upregulated at puberty and, not surprisingly, its absence in MMP-9 null mice has no effect on ductal development. The transmembrane MMP, MMP-14, regulates MMP-2 activation at the cell surface. Consequently, deletion of the MMP-14 protease from the mammary gland may also inhibit ductal development through regulation of MMP-2 activation. These animals exhibit retarded adipocyte differentiation within the mammary fat pad and we are currently evaluating whether epithelial alterations exist as well. Although we have shown that MMP-2 and MMP-3 have distinct roles during ductal development, the absence of either MMP-2 or MMP-3 fails to ablate ductal morphogenesis entirely indicating that other MMP family members are likely to have a role in these processes.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-7324 supported this work.

DEPARTMENT OF THE ARMY
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